

ISOLATION, CHARACTERIZATION AND MONITORING OF ANTIBIOTIC RESISTANCE IN *PASTEURELLA MULTOCIDA* ISOLATES FROM BUFFALO (*BUBALUS BUBALIS*) HERDS AROUND LAHORE

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ABSTRACT

Haemorrhagic septicaemia (HS) caused by *Pasteurella multocida* (*P. multocida*) is an important disease of buffaloes causing heavy economic losses in Pakistan. Samples from 20 HS suspected carcasses of buffaloes were processed and the recovered isolates were identified by cultural, morphological, biochemical and serological tests. All the isolates were also processed for mouse pathogenicity test and antibiogram assay. Sixteen isolates were identified as *Pasteurella multocida*. Serologically all isolates were associated with Carter's sero group B. Maximum number of isolates were recovered from samples of bone marrow, lungs and spleen. All the isolates were found pathogenic to mice. Antibiogram assay of all the fields isolates depicted the highest sensitivity 87.5% for ciprofloxacin, ofloxacin, enrofloxacin and gentamicin, 81.25% for norfloxacin and amikacin, 75% for kanamycin, 56.25% for tetracycline, 25% for chloramphenicol, doxycycline and vancomycin, erythromycin where as sulfadiazine (12.5%) was found least effective. No resistance was observed for ciprofloxacin, enrofloxacin, ofloxacin and norfloxacin where as maximum resistance was observed against erythromycin and sulfadiazine (50%). It is concluded that all *P. multocida* isolates belonged to Carter's group B and found sensitive to ciprofloxacin and ofloxacin.

Key words. Buffalo, *Pasteurella multocida*, Isolation, Antibiotic resistance.

INTRODUCTION

Hemorrhagic septicemia is an economically important bacterial disease of cattle and buffaloes (Chandrasekaran *et al.*, 1994) and buffaloes are more susceptible than cattle (DeAlwis, 1990). The disease remains a significant obstacle to sustainable livestock production in most parts of tropical Asia and Africa. It is caused by *Pasteurella multocida* a natural inhabitant of the mucosal surfaces of upper part of the respiratory tract of ruminants, and under predisposing environmental or management conditions which constitute stress for the animals such as transport (shipping fever), marketing, change of feed, climate or ventilation (Radostits *et al.*, 2000). The disease is per acute, having a short clinical course, involving severe depression, pyrexia, sub mandibular edema, and dyspnea, followed by recumbency and death (Horadagoda *et al.*, 2001). Diagnosis of the Pasteurellosis has been traditionally based on the clinical symptoms and isolation/identification of the causative organism. Antibiotics are used to a large extent for treatment of hemorrhagic septicemia. However, the prolonged and indiscriminate use of antibiotics has resulted in organism and even multi drug resistant (MDR) forms of *P. multocida* have emerged (Arora *et al.*, 2005; Shivachandra *et al.*, 2004). Antimicrobial resistance of

Pasteurella isolates varies according to the host animal species, time, geographical origin and antimicrobial pretreatment of the animals (Caprioli *et al.*, 2000). The aim of the present study was the isolation and identification of the pathogen from hemorrhagic septicemia suspected carcasses of buffaloes, which could be used for development of vaccine from local isolates to effectively prevent the disease in future, to determine the antimicrobial susceptibility status of field isolates of *Pasteurella multocida* that will assist practitioners in the rational selection of antimicrobial agents and make the prudent use of these drugs for control of the disease.

MATERIALS AND METHODS

Samples: Samples of blood, bone marrow and tissue specimens (pieces from liver, spleen and lung) were collected from HS suspected carcasses of 20 buffaloes from different areas around Lahore during the period from July, 2005 to June, 2006. The samples were processed for isolation and identification of suspected pathogen by standard method (Carter, 1984).

Isolation: Samples were inoculated on nutrient agar, blood agar (5% sheep blood) and MacConkey's agar. The smears were prepared from representative colonies and

microbes were characterized microscopically by using Gram's staining method.

Biochemical tests: Biochemical tests for all the isolates were performed. Peptone water grown culture of each isolate was inoculated in 1% glucose, sucrose, sorbitol, manitol, fructose, dulcitol, lactose, silicin, arabinose and maltose, incubated aerobically at 37°C for 72 hours. Indol, oxidase, catalase, urease production and nitrate reduction tests were carried out according to their standard bacteriological procedure (Carter, 1984).

A slide agglutination test using the antiserum against the capsular type B was also performed for each isolate (OIE, 1992).

Pathogenicity test: Pathogenicity of each isolate was tested in six weeks old Swiss albino mice. A total of six mice were used for each isolate. Mice were inoculated intra-peritoneally with 0.1 ml of inoculum containing 0.3×10^8 organisms per ml in sterile normal saline. Control mice were injected with 0.1 ml of sterile saline. All the mice were kept under observation and mortality was recorded. Blood smears were prepared from the heart blood of dead mice and stained with Giemsa stain. Re-isolation of *P. multocida* from heart blood of the dead mice was carried out on sheep blood agar (Buxton and Fraser, 1977).

Antibiogram assay: Each of the isolate was tested for sensitivity against 15 different antibiotics such as gentamicin, kanamycin, amikacin, tetracycline, doxycycline, vancomycin, erythromycin, sulfadiazine, amoxycillin, ampicillin, chloramphenicol, ciprofloxacin, norfloxacin, ofloxacin and enrofloxacin using the standard method of National Committee for Clinical Laboratory Standards (NCCLS, 1990). An eighteen hours culture of each isolate in Brain Heart Infusion broth was plated on Muller-Hinton agar medium enriched with 5% sheep blood. The culture was allowed to adsorb for 10 minutes and then the antibiotic discs (Oxoid) were placed on the plate at an appropriate distance from each other. The plates were incubated aerobically at 37°C for 24 hours. The diameters of inhibition zones surrounding the antibiotic discs were measured and subsequently matched with the standard inhibition zone diameters of respective antibiotic discs. On the basis of size of inhibition zones of various antibiotics, the isolates were classified as sensitive, intermediately sensitive or resistant.

RESULTS

Sixteen bacterial isolates were recovered from the samples collected from 20 HS suspected buffalo carcasses. All the isolates exhibited smooth glistening, translucent colonies on nutrient agar, failed to grow on MacConkey agar and produced non hemolytic dewdrop like colonies on sheep blood agar. Grams stained smears

from all isolates revealed microscopically gram negative bipolar coccobacilli.

All the isolates fermented glucose, sucrose, sorbitol, manitol, fructose, arabinose and maltose with acid production only. A negative reaction was observed for dulcitol, lactose and silicin. All the isolates were found positive for indol, oxidase, catalase production and nitrate reduction tests. All the isolates were negative for urease production. Cultural, morphological and biochemical characteristics identified all isolates as *Pasteurella multocida*. Serologically all isolates were found to be Carter's sero group B.

Sixteen samples (80%) out of 20 samples of each of the bone marrow, lung and spleen yielded pure growth of the causative agent where as 10 (50%) and 5(25%) out of 20 samples of each of the liver and blood yielded pure growth on sheep blood agar.

All the field isolates killed mice within 24 to 36 hours post inoculation. Giemsa stained smears prepared from heart blood of dead mice revealed bipolar organisms. From heart blood of mice colonies representative of *P. multocida* were isolated on sheep blood agar.

Table I. Antibiogram assay of sixteen field isolates of *P. multocida*

| Antibiotics | Sensitive | Intermediate | Resistant |
|-----------------|------------|--------------|-----------|
| Gentamicin | 14(87.5%) | 1(6.25%) | 1(6.25%) |
| Kanamycin | 12(75%) | 0 | 4(25%) |
| Amikacin | 13(81.25%) | 3(18.75%) | 0 |
| Tetracycline | 9(56.25%) | 2(12.5%) | 5(31.25%) |
| Doxycycline | 8(50%) | 5(31.25%) | 3(18.75%) |
| Vancomycin | 8(50%) | 4(25%) | 4(25%) |
| Erythromycin | 4(25%) | 4(25%) | 8(50%) |
| Sulfadiazine | 2(12.5%) | 6(37.5%) | 8(50%) |
| Amoxycillin | 4(25%) | 5(31.25%) | 7(43.75%) |
| Ampicillin | 4(25%) | 8(50%) | 4(25%) |
| Chloramphenicol | 8(50%) | 4(25%) | 4(25%) |
| Ciprofloxacin | 14(87.5%) | 2(12.5%) | 0 |
| Norfloxacin | 13(81.25%) | 3(18.75%) | 0 |
| Ofloxacin | 14(87.5%) | 2(12.5%) | 0 |
| Enrofloxacin | 14(87.5%) | 2(12.5%) | 0 |

Sixteen field isolates of *P. multocida* showed sensitivity to 15 antibiotics, as 87.5% isolates were found sensitive to ciprofloxacin, ofloxacin, enrofloxacin and gentamicin followed by 81.25% isolates to norfloxacin and amikacin. Seventy five percent isolates were sensitive to kanamycin. For tetracycline sensitivity was 56.25%. Fifty percent isolates were sensitive to chloramphenicol, doxycycline and vancomycin. Twenty percent isolates were sensitive to erythromycin. The lowest sensitivity (12.5%) was observed for sulfadiazine. Resistance was not observed for ciprofloxacin, enrofloxacin, ofloxacin and norfloxacin. However for

erythromycin and sulfadiazine resistance was 50% (Table I).

DISCUSSION

In clinical cases, diagnosis of hemorrhagic septicemia is made on the basis of clinical signs, gross pathological lesions, herd history, morbidity and mortality pattern. However confirmation of tentative clinical diagnosis needs isolation and identification of causative organism from the morbid material. In the present study, cultural, morphological and biochemical characteristics of all the isolates recovered from morbid materials were in accordance with those of *P. multocida* (Dutta *et al.*, 2001; Kumar *et al.* 2009).

Long bones from the suspected carcass of hemorrhagic septicemia were found to be the sample of choice for isolation of *P. multocida*. Isolation of *P. multocida* from the lung and spleen samples was comparable to that of long bones. *P. multocida* can be isolated from wide range of organs such as lung, kidney, heart, spleen, liver, brain, tonsils and lymph nodes (left and right retropharyngeal and pre-scapular nodes) from experimentally infected calves with *P. multocida* (OIE, 1992; Mark *et al.*, 2007). From the liver and blood samples, recovery of the causative agents was poor. Postmortem changes might have inactivated the causative agents in these samples.

All the field isolates of *P. multocida* were found pathogenic for mice and killed the mice within 6-24 hours post-infection. In the BALB/c mice, when experimentally infected through intra-peritoneal route, an overwhelming septicemia was observed within 30 hours post infection (Ramdani *et al.*, 1990).

Sixteen pure isolates of *P. multocida* were tested for their sensitivity against different antibiotics available for the treatment of bacterial infections in animals. All of the 16 isolates were found highly sensitive (87.5%) to ciprofloxacin, ofloxacin and enrofloxacin. Yoshimura *et al.* (2008) and Kumar *et al.* (2009) also found enrofloxacin the most effective antibiotic against *P. multocida*. Similarly, Shayegh *et al.* (2009) reported 100% sensitivity of the bacteria to ciprofloxacin and enrofloxacin. In present study no resistance was observed for quinolones. Similar observations were also recorded by Anwar *et al.* (2000). In contrast to our finding, Carty *et al.* (2005) observed the acquired resistance of *P. multocida* isolates to enrofloxacin. Among aminoglycosides, gentamicin was found highly effective (87.5%) against field isolates. Other authors found the efficacy of gentamicin against *P. multocida* as controversial (Verma *et al.*, 2004; Kumar *et al.*, 2009). Amikacin and Kanamycin were highly effective (81.25%), and (75%), respectively but Yoshimura *et al.* (2008) found aminoglycosides as less effective. Tetracycline, chloramphenicol, doxycycline and

vancomycin were moderately effective against the bacteria. Erythromycin and sulfadiazine were not very effective. These observations are in accordance to Watts *et al.* (1994) who frequently encountered resistance of *P. haemolytica* and *P. multocida* of bovine origin to erythromycin, and sulfamethazine.

In conclusion, all the field isolates were similar in cultural, morphological, biochemical and serological characteristics and can be used for development of HS vaccine to control the disease. The local isolates of HS showed increasing level of resistance to the antibiotics that are extensively used in field for treatment of the disease. It is therefore recommended to monitor the antibiotic sensitivity of *P. multocida* from time to time in future to design the effective regimen for treatment of the disease.

REFERENCES

- Anwar, M. A. H., S. U. Rahman and R. Ahmad (2000). Sensitivity of *Pasteurella multocida* isolated from cattle and buffaloes. *PJBS.*, 3(4):692-93.
- Arora, A. K., S. Virmani, S. K. Jand and M. S. Oberoi (2005). Isolation, characterization and antibiogram of *Pasteurella multocida* isolates from different animal species. *Indian J. Anim. Sci.*, 75: 749-752.
- Buxton, A. and G. Fraser, (1977). *Animal Microbiology*. Blackwell Scientific Publications Ltd. Oxford. London, UK.
- Caprioli, A., L. Busani, J. L. Martel and R. Helmuth (2000). Monitoring of antibiotic resistance in bacteria of animal origin: epidemiological and microbiological methodologies, *Int. J. Antimicrob. Agents.*, 14:295-301.
- Carter, G. R. (1984), *Diagnostic procedures in veterinary bacteriology and mycology*, 4th ed., Charles C Thomas Publishing Co., Springfield, IL.
- Catry, B., F. Haesebrouck, S. D. Vlieghe, B. Feyen, M. Vanrobaeys, G. Opsomer, S. Schwarz and A. D. Kruif, (2005). Variability in acquired resistance of *Pasteurella* and *Mannheimia* isolates from the nasopharynx of calves, with particular reference to different herd types. *Microb Drug Resist.*, 11(4):387-94.
- Chandrasekaran, S., L. Kennett, P. C. Yeap, N. Muniandy, B. Rani and T. K. S. Mukkur, (1994). Characterization of immune response and duration of protection in buffaloes immunized with hemorrhagic septicemia vaccines. *Vet. Microbiol.*, 41:213-219.
- De Alwis, M. C. L. (1990). *Haemorrhagic Septicaemia*. ACIAR Monograph., 57:36.
- Horadagoda, N. U., J. C. Hodgson, G. M. Moon, T. G. Wijewardana and P. D. Eckersall (2001). Role of endotoxin in the pathogenesis of

- haemorrhagic septicaemia in the buffalo. *Microb. Pathog.*, 30:171-178.
- Kumar, P., V. P. Singh, R. K. Agrawal and S. Singh, (2009). Identification of *Pasteurella multocida* isolates of ruminant origin using polymerase chain reaction and their antibiogram study. *Trop Anim Health Prod.*, 41:573–578.
- Manual of standards for diagnostic tests and vaccines, (1992). Office international des epizooties.
- Mark, P., J. Dagleish, C. Hodgson, S. Ataei, A. Finucane, J. Finlayson, J. Sales, R. Parton and J. G. Coote (2007). Safety and protective efficacy of intramuscular vaccination with a live avian derivative of *Pasteurella multocida* B:2 against experimental hemorrhagic septicemia in calves. *IAI.*, 75(12) : 5837-5844.
- NCCLS, (1990). Performance standards for antimicrobial disk susceptibility tests. 4th Ed., Villanova, PA, USA.
- Radostits O. M., C. Gay, D. C. Blood and K.W. Hinchcliff (2000). A textbook of the diseases of cattle, sheep, pigs, goats, and horses, 9th ed., Saunders, Philadelphia, London.
- Ramdani, H. J. S. Dawkins, R. B. Johnson, T. L. Spencer, and B. Adler (1990). *Pasteurella multocida* infections in mice with reference to haemorrhagic septicaemia in cattle and buffalo. *Immunology and Cell Biology*, 68: 57–61.
- Shayegh, J., P. Mikaili, J. D. Sharaf, and A. Rastgn (2009). Antimicrobial resistance evaluation of Iranian ovine and bovine *Pasteurella multocida*. *J. Anim. Vet. Adv.*, 8 (9):1753-56.
- Shivachandra, S. B., A. A. Kumar, A. Biswas, M. A. Ramakrishnan, V. P. Singh and S. K. Srivastava (2004). Antibiotic sensitivity patterns among Indian strains of Avian *P. multocida*, *Tropical Animal Health and Production*, 36:743–750.
- Verma, S. C., N. K. Mahajan, G. Malik and J. P. Dahiya, (2004). An epidemiological study on bovine hemorrhagic septicemia in Haryana, *Indian J. Anim. Sci.*, 38:14–19.
- Watts, J. L., R. J. Yancey, J. S. A. Salmon, and C. A. Case (1994). A 4-year survey of antimicrobial susceptibility trends for isolates from cattle with bovine respiratory disease in North America. *J. Clin. Microbiol.*, 32 (3):725-31 .
- Yoshimura, H., M. Ishimaru, Y. S. Endoh and A. Kojima (2008). Antimicrobial Susceptibility of *Pasteurella multocida* Isolated From Cattle and Pigs. *J. Vet. Med. Series B*, 48 (7):555–560.